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① Title of invention

1 Please give the title of the invention: ADJUVANT COMPOSITIONS

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② Applicant's details

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⑤ Claiming an earlier application date

5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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Yes ☐ No ☒ ➡ ***go to 6***

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Continuation sheets for this Patents Form 1/77			0
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ADJUVANT COMPOSITIONS

This invention relates to an adjuvant composition for stimulating or enhancing the protective immunogenic effects of an antigen co-administered therewith; and to a method of stimulating or enhancing the protective immunogenic effect of an antigen by co-administering therewith an adjuvant composition.

The majority of pathogenic microorganisms initiate infection by attaching themselves to the mucosal epithelial cells lining the gastro-intestinal, oropharyngeal, respiratory or genito-urinary tracts. Some pathogens, such as influenza virus, Bordetella pertussis, or Vibrio cholerae, may remain at or within the mucosal tissue, while others, such as Salmonella typhi or hepatitis A virus, possess mechanisms that allow them to penetrate into deeper tissues and spread systemically. The specific and non-

specific defence mechanisms of the mucous membranes provide first line protection against both types of pathogen. Non-specific effectors include resident macrophages, antimicrobial peptides, lactoferrin and lysozyme, extremes of pH, bile acids, digestive enzymes, mucus, shedding of epithelial cells, flushing mechanisms (peristalsis, ciliary beating, micturation, etc) and competition from local flora. However, successful pathogens have generally evolved means to survive the non-specific defences present at the site they infect and it is the secretory immune system which plays a major role in protecting against diseases caused by a number of bacterial and viral pathogens, and is probably the major effector against pathogens that are restricted to mucosal surfaces. For organisms that spread systemically, local and systemic immune responses are probably needed for optimum immunity.

A means of stimulating the local and systemic lymphoid tissues is needed for effective immunisation against many diseases. Unfortunately, current parenteral immunisation regimes often stimulate weak or undetectable secretory responses. In order to achieve efficient stimulation of the mucosa-associated lymphoid tissue (MALT), the immunogen needs to be applied topically to the mucosal surface during the course of vaccination. However, this is not as straightforward as it seems. Most non-replicating immunogens are poorly immunogenic when ingested or inhaled, and soluble proteins are particularly inefficient mucosal immunogens. This is undoubtedly because the non-specific

defences will readily denature, degrade and eliminate most soluble proteins resulting in the MALT encountering minute quantities of such immunogens.

Administering large repeated doses of a particular protein may be expected to enhance the immune response. However, the result of such immunisation is often the induction of a state of immunological unresponsiveness known as oral tolerance where the individual responds poorly to subsequent parenteral immunisation with the same antigen (it would be more accurately labelled mucosal tolerance because inhalation of large amounts of soluble proteins also induces this state). The regulatory mechanisms involved in initiation of oral tolerance are poorly understood but are believed to have evolved to prevent animals developing inappropriate and possibly deleterious immune response to environmental and dietary proteins. One of the major goals of modern vaccinology is to devise means of eliciting strong mucosal and systemic immune responses to soluble proteins.

Although many soluble proteins are poor mucosal immunogens, some proteins, particularly microbial components that can recognise and bind to receptors on the surface of eucaryotic cells, can elicit local and systemic immune responses. In particular cholera toxin (CT) is the most potent mucosal immunogen known. CT is also a mucosal adjuvant that greatly enhances the responses to co-administered antigens. In mice only minute quantities of CT are necessary for the adjuvant effect. Unfortunately,

a dose of 2µg of CT fed to human volunteers is diarrheaogenic and a dose of 5µg induced purging indistinguishable from classical cholera. Active CT is therefore clearly unacceptable as a mucosal adjuvant in humans.

CT is a bipartite toxin consisting of a protomer (CTA) and a B pentamer (CTB). CTA is the enzymatic moiety of CT responsible for the covalent modification of host G proteins and the consequent toxicity of CT. CTB, which mediates the binding of CT to its receptor (ganglioside GM_1) on the surface of eucaryotic cells, is non toxic and is also a good mucosal immunogen. CTB has been investigated as a mucosal adjuvant by many groups with conflicting results. CTB obtained from commercial suppliers is prepared from CT and often contains trace quantities of CT which could be responsible for the adjuvanticity of CTB reported by some authors. Furthermore, it has been found that recombinant CTB and the highly related E. coli heat labile toxin B pentamer (LTB), although immunogenic themselves, are devoid of adjuvanticity. Thus, Holmgren et al (Vaccine, Vol II, pp 1179-1184, 1993) have reported that when highly purified or recombinant CTB was used, they were consistently unable to observe an adjuvant action of CTB for other antigens admixed therewith. Holmgren et al concluded that the whole CT molecule is required for the adjuvant action. They also tested a mutant form of E. coli heat labile toxin (LT) in which the B sub-unit was identical to that of the normal B sub-unit, and the A sub-

unit was identical with the exception of a single amino acid substitution in position 112 (Glu → Lys). However, the mutant form, which did not possess ADP-ribosylating activity and did not cause fluid secretion in rabbit ligated loops, failed to give rise to any significant IgA response against itself and demonstrated no adjuvant properties.

Pertussis toxin (PTX), like CT, has an AB_5 structure. Both CT and PTX are ADP-ribosylating toxins but they have different cellular receptors and substrates. PTX can produce a myriad of biological effects and is one of the major protective antigens of B.pertussis, and inactivated forms of PTX form the basis of current and experimental acellular pertussis vaccines.

Pertussis toxin (PTX) has been reported to have adjuvant properties, but a major drawback, in addition to its inherent toxic properties is its property of stimulating and enhancing IgE production thereby leading to anaphylaxis to co-administered proteins - see for example Mu et al Infection and Immunity, pp. 2834-2840, July 1993.

Thus, there remains a need for a mucosal adjuvant which lacks the toxic and undesirable side effects described above.

It has now been found that a particular mutant form of pertussis toxin is not only lacking in the toxic properties of the wild type toxin, but is an excellent adjuvant, as well as displaying good immunogenic properties itself.

Accordingly, in a first aspect, the invention provides

a method of stimulating or enhancing a protective immune response to an antigen in a mammal which method comprises co-administering with the antigen an effective adjuvant amount of a non-toxic mutant form of pertussis toxin in which the Glu 129 amino acid in the S_1 sub-unit has been substituted by another amino acid, e.g. glycine.

In another aspect, the invention provides a vaccine composition comprising a first antigen and an effective adjuvant amount of a non-toxic mutant form of pertussis toxin in which the Glu 129 amino acid in the S_1 sub-unit has been substituted by another amino acid.

In a further aspect, the invention provides the use of a non-toxic mutant form of pertussis toxin in which the Glu 129 amino acid in S_1 sub-unit has been substituted by another amino acid, for the manufacture of an adjuvant composition for stimulating or enhancing the protective immune response of an antigen co-administered therewith.

The non-toxic mutant form of pertussis toxin preferably is a double mutant form; for example, a double mutant form in which the arginine 9 amino acid residue in the S_1 sub unit has been substituted, e.g. by lysine.

Particular examples of non-toxic double mutant pertussis toxins for use in the present invention are those disclosed in European Patent Application EP-A-0462534 (Sclavo SpA). A preferred non-toxic double mutant toxin is the mutant described in Example 1 of EP-A-0462534, in which the arginine 9 residue has been substituted by lysine, and the glutamic acid 129 residue has been substituted by

glycine. This mutant is referred to hereinafter as PT 9K/129G.

An example of an antigen that may be co-administered with the mutant pertussis toxin and whose immunogenicity is markedly enhanced when thus co-administered is the C-fragment of tetanus toxin (hereinafter referred to as Frg C).

The antigen whose immunogenic activity is to be stimulated or enhanced, and the adjuvant pertussis mutant toxin, are preferably administered mucosally. Thus they may be administered orally, but it is preferred that the antigen and the mutant toxin be given by intranasal administration.

The antigen and the adjuvant mutant toxin may be administered separately, for example, within a short period of one another, or they may be administered together simultaneously. When administered together, they may be formulated as a mixture of discrete entities. Alternatively, they may be chemically linked together, or may form part of a fusion protein produced by recombinant DNA methods.

In a particular aspect of the invention there is provided a method of immunising a host such as a mammal, (e.g. human) against infection, which method comprises administering an effective amount of an antigen capable of eliciting an immune response against said infection and an effective adjuvant amount of the mutant pertussis toxin as hereinbefore defined directly to a mucosal surface in the

host to stimulate or enhance in said mucosal surface the immune response to the said antigen.

The compositions of the present invention preferably are formulated as an aqueous solution for administration as an aerosol or nasal drops, or as a dry powder, e.g. for inhalation.

Compositions for administration as nasal drops may contain one or more excipients of the type usually included in such compositions, for example preservatives, viscosity adjusting agents, tonicity adjusting agents, buffering agents and the like. The antigen typically is selected such that it is non-toxic to a recipient thereof at concentrations employed to elicit an immune response.

The invention will now be illustrated, but not limited, by reference to the examples set forth below, and accompanying Figures in which:-

Figure 1 illustrates the effect of cholera toxin (CT), pertussis toxin (PTX) and PT-9K/129G on the serum IgG response to Fragment C of tetanus toxin;

Figure 2 illustrates the serum IgG response to PTX, PT-9K/129G and CT in mice immunised intranasally;

Figure 3 illustrates the effect of CT, PTX and PT-9K/129G on the serum IgG response to Fragment C, and the effect of boosting;

Figure 4 illustrates the serum response to PTX, PT-9K/129G and CT in intranasally immunised mice and the effect of boosting;

Figure 5 illustrates the effect of CT, PTX and PT-

9K/129G on the secretory IgA response to Fragment C in the respiratory tract of NIH:S mice;

Figure 6 illustrates the secretory IgA responses to CT, PTX and PT-9K/129G in the respiratory tract of NIH:S mice;

Figure 7 illustrates the serum anti-Frg C response in BALB\c and NIH:S mice; and

Figure 8 illustrates the serum anti-PTX and PT-9K/129G IgG responses in BALB\c and NIH:S mice.

EXAMPLE 1

Serum and secretory immune responses to fragment C, PT, PT 9K/129G and CT in intranasally immunised mice.

Groups of adult female outbred NIH:S mice were immunised intranasally (I\N) with 10µg of fragment C (FRG C) alone or admixed with 5µg of cholera toxin (CT), active pertussis toxin (PTX) or PT-9K/129G 25d apart. The cholera toxin was obtained from Sigma (Dorset, UK); the active pertussis toxin was obtained from Calbiochem (Nottingham, UK) or NIBSC (Herts., UK); and the mutant pertussis toxin PT-9K/129G was obtained from IRIS (Siena, Italy).

Prior to immunisation, the mice were anaesthetised with metathane and the antigen was added to the external nares of the mice as they recovered consciousness. Antigen was taken into the respiratory tract by inhalation.

As a comparison, a group of mice was immunised twice subcutaneously (S\C) with 10µg of fragment C adsorbed to

aluminium hydroxide gel (alhydrogel). Serum samples were taken 14 days after primary immunisation and serum and nasal wash samples 14 days after the boost. Antibody responses against each of the components were determined by ELISA.

The serum anti-Frg C IgG responses following a single immunisation are depicted in Figure 1, in which each point on the graph represents the mean value obtained from 5 mice. Anti-fragment C antibodies were not detected in mice immunised I\N with Frg C or Frg C + PTX. In contrast mice receiving Frg C combined with PT-9K/129G or CT had significant amounts of anti-Frg C antibodies in their serum. The levels of anti-Frg C antibodies were similar in the two groups and were somewhat lower than those in parenterally immunised mice. Mice immunised I\N with Frg C and CT mounted a very strong serum IgG response to CT (titres greater than 14580, Fig. 2). Anti-PTX antibodies were present in mice receiving PT-9K/129G but not PTX Figure 2).

Following boosting, some of the mice that received Frg C alone I\N seroconverted (2/5). Mice in the Frg C + PTX group also exhibited an Frg C response following the second dose and this was greater than that of mice given Frg C alone I\N (Figure 3) indicating that PTX had acted as an adjuvant. Also the boosted Frg C + PTX mice had developed circulating anti-PTX antibodies (Figure 4). However, both the anti-Frg C and anti-PTX response were considerably inferior to that of Frg C + PT-9K/129G mice (Figures 3 and

4). The greatest responses were seen in the Frg C + CT group. The anti-Frg C response was greater than in S\C immunised mice, anti-Frg C IgG could still be detected at a serum dilution of 1/800,000. At the equivalent dilution the anti-CT response had not begun to titrate.

EXAMPLE 2

Respiratory IgA responses

The secretory responses were studied in the nasal lavages of mice immunised twice as described in Example 1. IgA anti-Frg C was present in the nasal lavage of all the mice in the Frg C + PTX, Frg C + PT-9K/129G and Frg C + CT groups (Figure 5). As in the serum, the response was greatest in the mice that received CT as adjuvant. There was very little Frg C specific IgA recovered from the nasal cavities of the mice given Frg C only I\N or parenterally. In each group a single mouse exhibited evidence of an IgA response and that was only detectable in undiluted nasal lavage. The corresponding IgA responses to PTX, PT-9K/129G and CT were stronger than those against Frg C and again the anti-CT response was the strongest (Figure 6).

EXAMPLE 3

Comparison of the intranasal immunogenicity and adjuvanticity of PTX and PT-9K/129 in inbred and outbred mice.

To confirm whether the difference observed between PTX

and PT-9K/129G in terms of immunogenicity and adjuvanticity was related to the source of the PTX, the single dose study in NIH:S mice was repeated using PTX from a different supplier (NIBSC). Also in order to examine whether the genetic background of the host influences the adjuvant and immunogenicity of active and genetically inactivated PTX the responses in an inbred strain (BALB\c) were studied as well. BALB\c mice were selected because it has previously been reported that this strain can mount a serum response, albeit weak, to parenterally administered pertussis toxin. Mice were immunised with I\N 10µg of Frg C alone or combined with PTX or PT-9K/129G as previously described. Blood samples were taken 15 days later and, to determine whether mice had developed protective immunity, they were challenge with tetanus toxin 22 days after immunisation and fatalities recorded for 4 days.

In both BALB\c and NIH:S mice the presence of PT-9K/129G provoked high titre antibodies to fragment C (Figure 7). In contrast to the previous study, PTX did have an adjuvant effect on the Frg C serum response in NIH:S mice and also did so in BALB\c mice. In both strains of mice, the combination of Frg C and PT-9K/129G induced a superior serum Frg C response compared to Frg C + PTX, although the difference was not large. In BALB\c mice, where comparison was made, the combination of Frg C and PT-9K/129G given I\N was nearly as effective as S\C immunisation with Frg C adsorbed to alhydrogel at eliciting a serum response. As previously, NIH:S mice did not

respond to a single 10µg dose of Frg C I\N. One of the three BALB\c mice immunised I\N with Frg C alone did mount a significant serum response, and this accounts for the large error bars in Figure 7, and also the protection data (see below).

Both strains of mice mounted similar serum responses to PTX and PT-9K/129G (Figure 8). The PTX response was measurable but weak. The PT-9K/129G response was greater by several orders of magnitude, as was found in earlier study.

Mice were challenged with tetanus toxin to determine whether the anti-fragment C antibodies elicited by I\N immunisation were protective, and the results are shown in Table 1. All of the mice receiving Frg C + PTX or PT-9K/129G were protected. The single BALB\c mouse that seroconverted following I\N immunisation with Frg C alone was protected, the remaining BALB\c mice in this group, the similarly immunised NIH:S mice and the naive control BALB\c mice all died.

TABLE 1

Comparison of serconversion to fragment C and protection from tetanus challenge in I\N immunised BALB\c and NIH:S mice.

	BALB\c				
	Frg C	Frg C + PTX	Frg C + PT-9K\129G	Frg C sub\cut	Naive
Seroconversion (positive\totals)	1/3	3/3	3/3	3/3	0/3
Protection (survivors/totals)	1/3	3/3	3/3	3/3	0/3
	NIH:S				
Seroconversion (positive\totals)	0/3	3/3	3/3	ND	0/3
Protection (survivors/totals)	0/3	3/3	3/3	ND	0/3

Mice were immunised I\N with a single dose of Frg C, Frg C + PT, Frg C + PT-9K/129G, Frg C + CT, or Frg C S\C absorbed to alhydrogel. They received 10µg of Frg C and 5µg of the other proteins. Mice were sample bled 15 days after immunisation and challenged 22 days after immunisation with 10 LD50 of tetanus toxin and deaths were records for 4 days.

CLAIMS

1. A method of stimulating or enhancing an immune response to an antigen in a mammal; which method comprises co-administering with the antigen an effective adjuvant amount of a non-toxic mutant form of pertussis toxin in which the Glu 129 amino acid in the S_1 sub-unit has been substituted by another amino acid.

2. A method according to Claim 1 wherein the antigen and the non-toxic mutant form of pertussis toxin are administered to a mucosal surface of the mammal.

3. A method according to Claim 2 wherein the antigen and the non-toxic form of pertussis toxin are administered intranasally.

4. A method according to any one of the preceding Claims wherein the antigen and the non-toxic mutant forms of pertussis toxin are administered at the same time.

5. A method according to Claim 4 wherein the antigen and the non-toxic mutant form of pertussis toxin are present in admixture in a composition administered to the mammal.

6. A method according to any one of the preceding Claims

glycine.

7. A method according to any one of the preceding Claims wherein the non-toxic mutant form of pertussis toxin is a double mutant in which the arginine 9 amino acid residue has been substituted by another amino acid.

8. A method according to Claim 7 wherein the arginine 9 amino acid has been substituted by lysine.

9. A method according to any one of the preceding Claims wherein the antigen is the C-fragment of tetanus toxin, or one or more immunogenic fragments thereof.

10. A vaccine composition comprising an antigen capable of eliciting a protective immune response in a mammal to which the composition is administered, and an adjuvant; characterised in that the adjuvant is a mutant form of pertussis toxin as defined in any one of the preceding Claims.

11. The use of a mutant form of pertussis toxin as defined in any one of the preceding Claims for the manufacture of an adjuvant composition for stimulating or enhancing a protective immune response of an antigen co-administered therewith.

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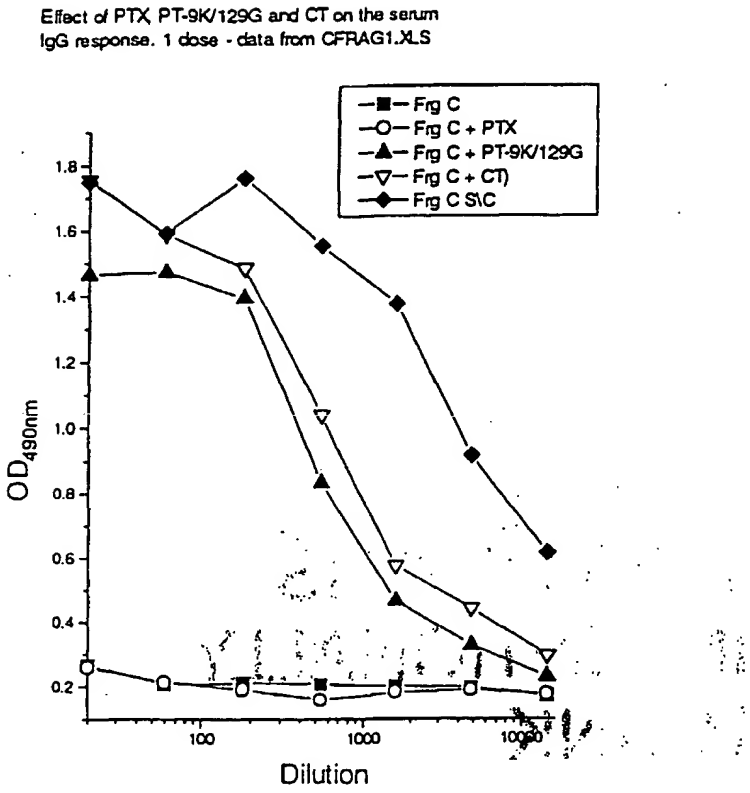

Figure 1. Effect of CT, PTX and PT-9K/129G on the serum IgG response to Frag C. Mice were immunised I\N with either Frag C + PTX, Frag C + PT-9K/129G, Frag C + CT or S\C with Frag C adsobed to alhydrogel. 10µg of Frag C was administered in each case and 5µg of PTX, PT-9K/129G or CT. Serum samples were obtained 14d after immunisation. The IgG responses were analysed by ELISA. Each point represents the mean of 5 mice.

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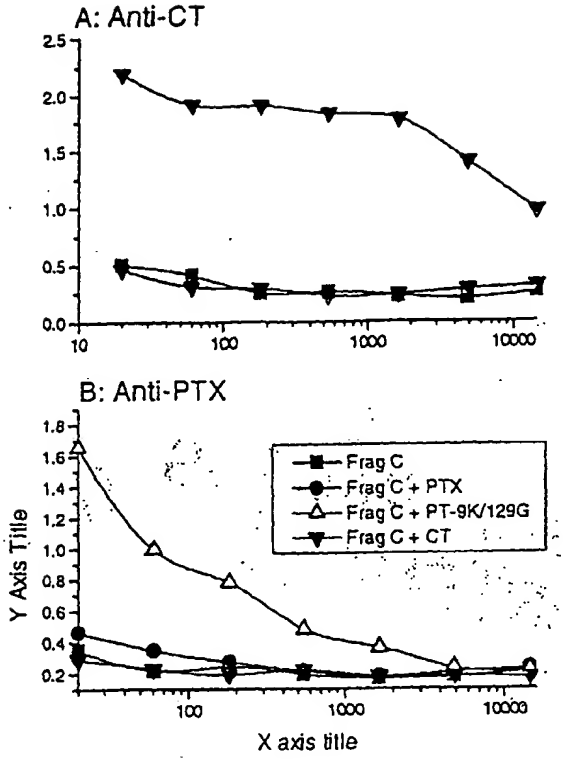

Figure 2. Serum IgG response to PTX, PT-9K/129G and CT in I\N immunised mice. Immunisation is described in Fig. 1. A, anti-CT; B, anti-PTX. Serum samples were obtained 14d after immunisation. The IgG responses were analysed by ELISA. Each point represents the mean of 5 mice.

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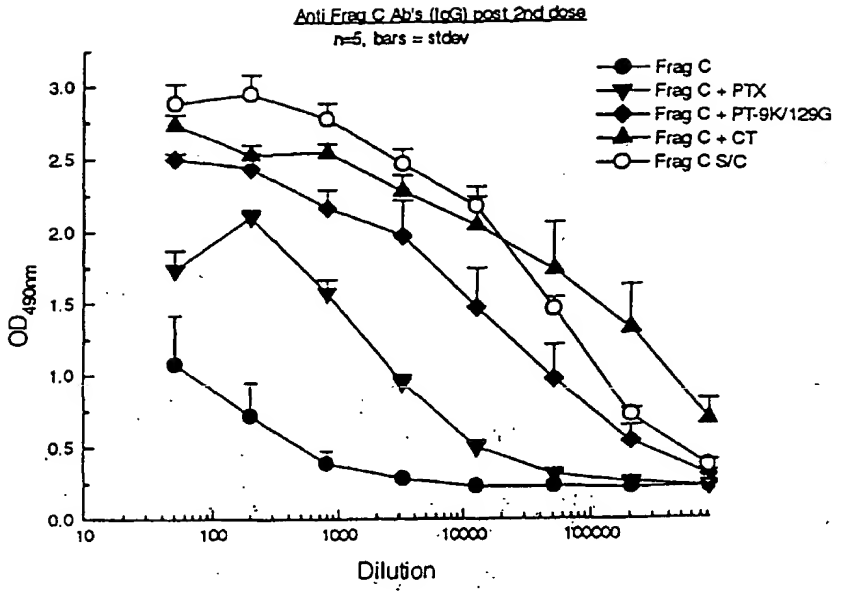

Figure 3. Effect of CT, PTX and PT-9K/129G on the serum IgG response to Frag C: Effect of boosting. Mice were immunised twice I\N with either Frag C + PTX, Frag C + PT-9K/129G, Frag C + CT or S\C with Frag C adsobed to alhydrogel. 10μg of Frag C was administered in each case and 5μg of PTX, PT-9K/129G or CT. Serum samples were obtained 14d after the second immunisation. The IgG responses were analysed by ELISA. Each point represents the mean of 5 mice + 1 SEM.

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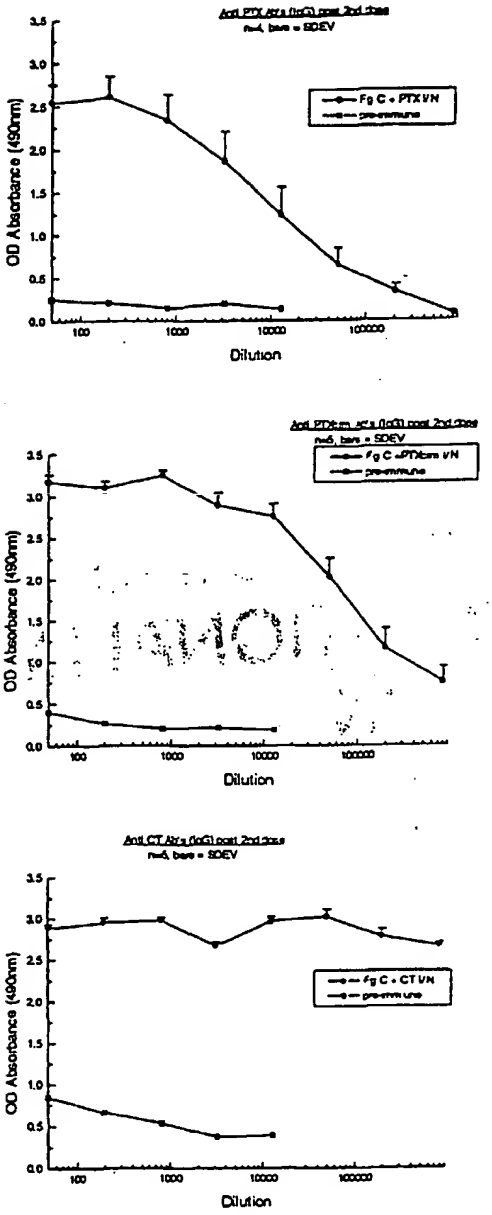

Figure 4. Serum anti-PTX, PT-9K/129G and CT in intransally immunised mice: the effect of boosting. Mice were immunised As described in Fig. 3. Serum samples were taken 14d after the boost and analysed by ELISA. Each point represents the mean of 5 mice + 1 SEM.

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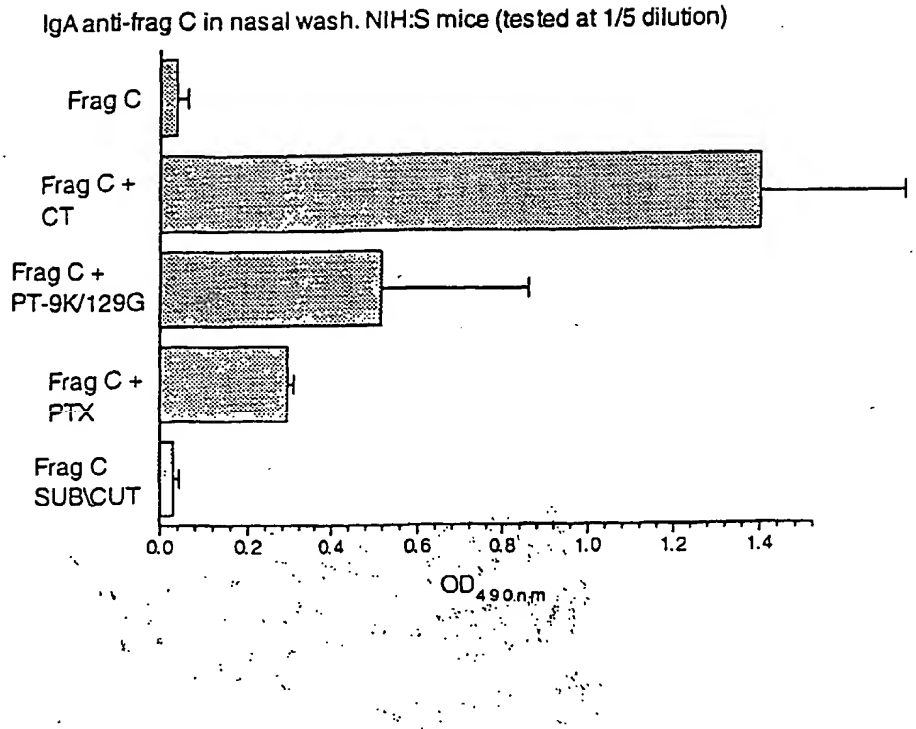

Figure 5. Effect of CT, PTX and PT-9K/129G on the secretory IgA response to Frag C in the respiratory tract of NIH:S. Mice were immunised as described in Fig. 3 and the nasal were lavaged passages 14d after the second immunisation. The IgA responses were analysed by ELISA. The reponse in a 1/5 dilution of nasal wash, is shown. Each bar represents the mean of 5 mice + 1 SEM.

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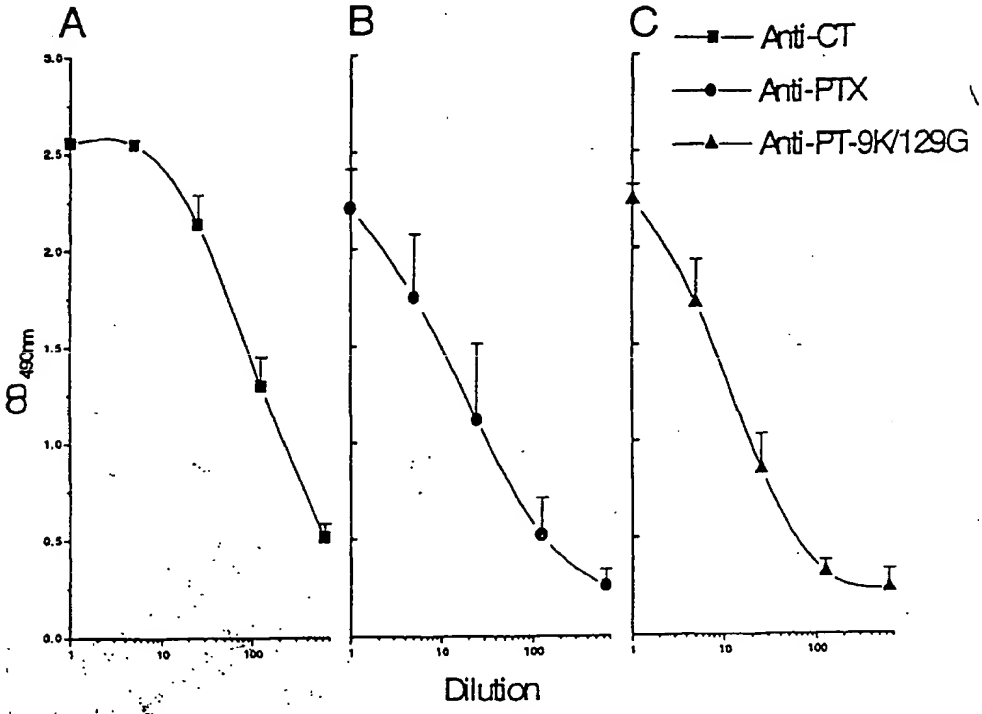

Figure 6. Secretory IgA responses to CT, PTX and PT-9K/129G in the respiratory tract of NIH:S. Mice were immunised as described and the nasal were lavaged passages 14d after the second immunisation. A, anti-CT; B, anti-PTX; C, anti-PT-9K/129G . Each point represents the mean of 5 mice + 1 SEM.

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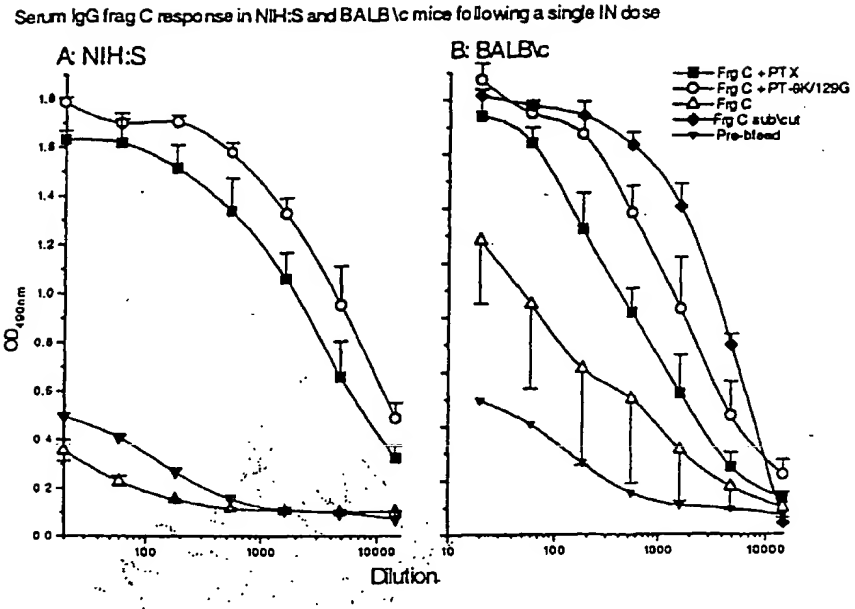

Figure 7. Serum anti-Frg C response in BALB\c and NIH:S mice. Mice were immunised IN with a single dose of Frg C, Frg C + PTX, Frg C + PT-9K/129G or Frg C absorbed to alhydrogel sub\cut. Serum samples were taken 18d later and analysed by ELISA. A, NIH:S mice; B, BALB\c mice. Each point represents the mean of the response of 3 mice + SEM

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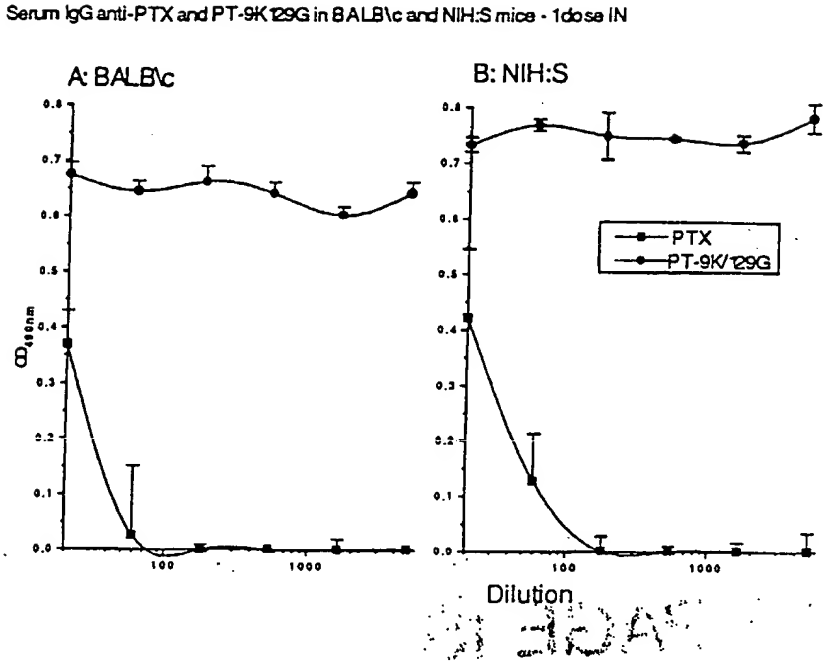

Figure 8. Serum anti-PTX and PT-9K/129G IgG responses in BALB/c and NIH:S mice. Mice were immunised IN with a single dose of Frg C + PTX or PT-9K/129G and serum samples were taken 18d later and analysed by ELISA. A, BALB\c mice; B, NIH:S mice. Each point represents the mean of the response of 3 mice + SEM.

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